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13. ABSTRACT (Maximum 200 words) Our biodefense research is primarily concerned with two inter-related issues 1) development of novel schemes for improving chemotaxonomic characterization of microbial pathogens and 2) mass spectrometry-based methods for trace detection of chemical markers for key agents in complex environmental matrices. Development of chemotaxonomic schemes for correct identification of species of bacteria relevant to the biodefense program is essential. The research employed a systematic search for new chemical markers employing state-of-the-art chemical and molecular approaches. A scheme for characterization of brucella (following our earlier work with bacilli) was developed. The feasibility of detecting markers for bacteria in environmental samples was demonstrated by developing a working method for detection of muramic acid (universally found in bacteria) in airborne dust. A prototype approach for the rapid (< 10 minutes) detection/identification of microorganisms (<i>B. anthracis</i>) based upon the combination of polymerase chain reaction (PCR) and electrospray mass spectrometry is also described. Extrapolation of this approach to environmental monitoring would represent a major improvement over existing technologies for biodefense.				
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(1) LIST OF MANUSCRIPTS

1. Wunschel D., Fox K., Fox A., Bruce J., Muddiman D. and Smith R. Analysis of double stranded polymerase chain amplification products from the *B. cereus* group by electrospray ionization fourier transform ion cyclotron resonance mass spectrometry. Rapid Commun. Mass Spectrom. 10: 29-35. 1996.
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(2) SCIENTIFIC PERSONNEL

Gavin Black received his Ph.D. in 1995 and was a post-doctoral fellow at ERDEC before leaving for a position at George Washington University. David Wunschel received his Ph.D. in Jan 1996 and is currently a post-doctoral fellow at PNNL. Mark Krahmer received his Ph.D. in July 1998 and continues as a post-doctoral fellow in our group. Three other students are currently working on the project, Yvette Johnson, Mike Kozar and Jim Walters. Mike Kozar is a Captain in the Army and on an Army fellowship. Other personnel fully or partially supported by the project at various times (Davis Harellson [past], Kelly Kim [past] and Paul Steinberg [currently]. Madan Nagapl and Karen Fox are both Research Associate Professors working on the project.

(3) REPORT OF INVENTIONS

1. Automated evaporator for chemical analysis. Patent number 5514336. Issued May 7 1996.
2. Automated derivatization of chemical samples. A. Fox and P. Steinberg. Patent application submitted, 1998.

(4) SCIENTIFIC PROGRESS AND ACCOMPLISHMENTS

There is great interest in an approach for the real-time detection of potential biological warfare agents in environmental specimens. Ideally, such a method should be fast (<~15 min), highly specific, sensitive and flexible for use against new threats. Fortunately, there are only a limited number of organisms that are likely BW agents. Criteria for BW agents (e.g., *Bacillus anthracis*, the causative agent of anthrax) include high virulence for man, ease of production, infection by the respiratory route and ability to survive under adverse environmental conditions.

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However, a universal instrumental method should be sufficiently flexible to be adaptable to identification of multiple and previously unknown threats including bioengineered agents.

Dr. Alvin Fox, is Professor of Bacteriology, USC School of Medicine and was appointed the Editor-in-Chief of the Journal of Microbiological Methods in January 1998. He has been part of the ARO/ERDEC Biodetection program since 1985. A major focus of this program is "Rapid detection of biological agents in environmental samples using mass spectrometry"; a problem whose solution is reaching fruition. Dr. Fox was among a group that introduced the use of gas chromatography-mass spectrometry GC-MS (Fox, et al 1980) and more recently gas chromatography-tandem mass spectrometry GC-MS/MS (Fox et al, 1996) and liquid chromatography-tandem mass spectrometry LC-MS/MS (Shahgholi et al, 1997) for non-culture based detection of bacterial markers in environmental samples. A more adaptable instrumental technique, coupling the polymerase chain reaction with mass spectrometric detection has the potential to achieve the same result in "real-time" and provide bacterial identification to the species level.

A. Trace detection of muramic acid, a universal marker for bacteria, in airborne dust using gas chromatography-tandem mass spectrometry. Muramic acid, a sugar constituent of bacterial cell wall peptidoglycan (PG) is not found elsewhere in nature and has been used as a measure of the levels of bacteria in a variety of matrices. Muramic acid is a reliable marker for peptidoglycan present in airborne dust. A procedure for the trace detection of bacterial peptidoglycan was first described in 1980 by the P.I. and adapted to air analysis first using GC-MS and later state-of-the-art GC-MS-MS. In the first GC-MS-MS study, a triple quadrupole instrument was used for trace analysis of muramic acid (as an alditol acetate derivative) in organic dust. More modestly priced and user friendly ion trap GC-MS-MS instruments subsequently became commercially available. We have demonstrated that results on an ion trap GC-MS-MS are of similar quality to those obtained with a triple quadrupole instrument (Fox et. al, 1996). Use of compact, simple to use MS/MS ion trap instrumentation is a major focus of the biodetection program.

Dust can be readily collected in mg amounts from air conditioners or household surfaces. However, the concentration of dust in air is often at the $\mu\text{g}/\text{m}^3$ level. This is clearly a trace analysis problem. Bacteria generally constitute only a small component of the dust. In order to test the practicality of monitoring a prototype environment was selected. Multiple samples were collected in a stable and a dairy over a period of 8 months. Filters were weighed before and after sampling and weight of dust determined. Teflon filters were acid heated to release muramic acid monomer from the polymeric matrix which was derivatized and analyzed by GC-MS/MS. The mean concentration of muramic acid in air over an 8 month period was $1.6 \pm 0.2 \text{ ng}/\text{m}^3$. This translates to a total bacterial load of $6.4 \pm 0.8 \times 10^3 \text{ c.f.u.}$ The mean total bacterial load (from

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plating) was $2.4 \pm 0.4 \times 10^5/\text{m}^3$ of air. Most colonies consisted of Gram positive bacilli and filamentous bacteria (Krahmer et. al., in press).

This GC-MS-MS technology provides a universal; approach for detection of bacteria. However, it is time consuming since the sugar marker must be released from the polymeric backbone. Derivatization (the conversion of the compound to a volatile form suitable for GC-MS analysis) is also slow. Finally, the analysis requires a chromatographic step which is also time consuming. An LC-electrospray MS-MS procedure for detection of muramic acid in dust was also developed. Although this procedure eliminated derivatization it is still too slow to be used for biodetection because of the hydrolysis step noted above. However, this does demonstrate the feasibility of using LC-MS/MS for detection of other larger molecules (e.g. proteins or nucleic acids) in environmental matrices. Analysis of these macromolecules would be much faster since hydrolysis is not necessary (Shahgholi et. al., 1997).

B. Universal species identification in "real-time" using polymerase chain amplification - electrospray tandem mass spectrometry. In the period 1994-97 we introduced a radically different approach for rapid detection of BW agents at the species level. The polymerase chain reaction (PCR) is an established alternative to culture for trace generation of molecular markers for pathogens in clinical and environmental specimens. 30 cycles of amplification can be achieved with commercial instrumentation in under 10 min. The rate-limiting step with conventional molecular biology approaches is the detection of these PCR products by gel electrophoresis (which generally takes several hr). Alternatively, MS analysis can be accomplished in a few sec.

Our initial work involved the use of a high resolution electrospray ionization (ESI) Fourier transform ion cyclotron mass spectrometry (FTICR) instrument. The MW of each of the strands of a double stranded PCR product was determined. The accuracy of the MW determination allowed recognition of single base substitutions, deletions and additions (see Figure 1). The 7 Tesla FTICR instrument used in studies described above was developed in Dr. Richard Smith's laboratory (Pacific Northwest National Laboratory) and it's capability exceeds that of many commercial instruments. Such state-of-the-art instruments are, however, not currently amenable to routine analysis with large throughput. It is important to demonstrate that PCR products can be analyzed on a commercial quadrupole MS instrument.

Synthetic oligonucleotides (30 and 89 mers) and polymerase chain reaction products of varying MW (62, 88, 89, and 114 base pairs [bp]) were analyzed by ESI using a quadrupole MS. The mass accuracy for nucleic acids in the 30-62 bp range was shown to allow determination of nucleotide substitutions and additions/deletions. For higher MW PCR products (88-114 bp) the mass accuracy of ESI-MS distinguishes single or multiple nucleotide insertions/deletions. In addition, ESI-quadrupole MS allows determination of MW of both strands of higher MW ds

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PCR products and can distinguish nucleotide modifications (e.g. with biotin).

Development of an instrumental approach coupling sample collection, polymerase chain amplification and mass spectrometry would allow rapid detection of BW agents in environmental samples.

C. Characterization of BW agents and their differentiation from other related organisms

In earlier published work, performed with ARO support, a scheme for identification of *B. anthracis* the number one BW agent was developed by determination of carbohydrate profiles and characterization of the interspace region (ISR) in the 16S- 23S RNA operon. We continue to focus on the interspace region in the 16S- 23S RNA operon since it is present universally in all bacterial pathogens. However since there is limited selection pressure on the interspace region, there is great diversity on the sequence and nucleotide length of this genomic region. However, the genes on either side of the spacer region (16S and 23S rRNA respectively) contain several conserved regions. Accordingly using primers against two conserved regions (in the 16S and 23S rRNA), different lengths/sequences of 16S-23S rRNA interspace region are generated for each characteristic BW agent. Experience gained with one group of BW agents is readily applied to other groups.

Strains W23 and 168 represent two distinct genetic clusters within the species *Bacillus subtilis*. *B. atrophaeus* (*B. subtilis* strain var. *niger*) was selected as a member of a group of species closely related to *B. subtilis*. Furthermore, *B. atrophaeus* is commonly used as a simulant for the number one BW agent (*B. anthracis*). The sequence of an ISR from *B. atrophaeus* var. *niger* is quite distinct from any of the ISRs found in *B. subtilis*. It is worthy of note that *B. subtilis* and *B. atrophaeus* are also both quite distinct from *B. anthracis*.

Molecular and chemical characteristics often provide complementary information in differentiation of closely related organisms. Identification of the 4 species pathogenic for man (*B. melitensis*, *B. abortus*, *B. suis* and *B. canis*) is problematic for many clinical laboratories depending primarily on serology and phenotypic characteristics. *B. melitensis* is one of a small group of bacterial pathogens suitable for use as a BW agent. PCR amplification of the 16S/23S rDNA interspace region was evaluated for species-specific polymorphism. *B. abortus*, *B. melitensis*, *B. suis* and *B. canis* produced identical PCR interspace profiles. However, these PCR products were unique to brucellae allowing them to be readily distinguished from other gram-negative bacteria (including *Bartonella* and *Agrobacterium*). Carbohydrate profiles differentiated *B. canis* from the other 3 *Brucella* species due to the absence of the rare aminosugar quinovosamine. PCR of the rRNA interspace region is useful in identification of the genus *Brucella* whilst carbohydrate profiling is capable of differentiating *B. canis* from the other *Brucella* species

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(5) TECHNOLOGY TRANSFER

Dr. Fox has collaborated with several analytical chemistry laboratories who perform DOD-related biodetection research in the area of electrospray ionization mass spectrometry: Dr Snyder (ERDEC), Dr. John Callahan (NRL) and Richard Smith, DOE. Dr. Fox's expertise in chemical markers for bacteria has been extremely helpful in focusing projects to study specific problems in microbiology and biodetection.